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Biosynthesis of Streptolydigin: Origin of the Oxygen Atoms

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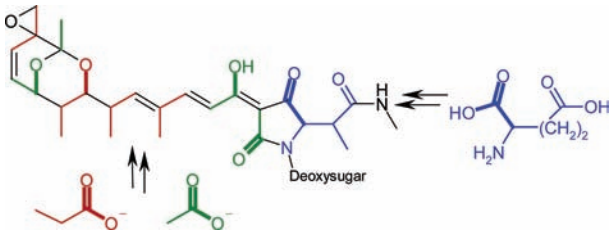
ABSTRACT



Biosynthetic incorporations of [^{13}C,^{18}O]-labeled precursors into streptolydigin reveal the origin of the oxygen atoms, limiting the possible pathways for acyltetramic acid ring assembly and for formation of the bicyclic ketal.

The acyltetramic acids (ATAs) are a group of natural products which feature a 2,4-pyrrolidinedione moiety with an acyl group on C-3 (C-3′ in **1**).[1] Examples include the RNA polymerase inhibitor streptolydigin (**1**),[2] as well as equisetin (**2**),[3] tenuazonic acid (**3**),[4] and lipomycin (**4**)[5] (Figure 1). The acyl groups range from acetyl in **3** to complex chains such as that bearing the intriguing bicyclic ketal in **1**. Other metabolites such as pramanicin (**5**),[6] fusarin C (**6**),[7] and talaroconvolutin (**7**)[8] are also structurally similar to ATAs.

Many biosynthetic studies have shown that the C-3 acyl group in ATAs is formed from a polyketide.[9] For example, Rinehart has shown that acetate and propionate label the acyl chain in **1** (C-1−C-14).[10,11] The pyrrolidinedione moiety of ATAs is formed from a C_2 unit (C-2′ and C-3′ in **1**), and the nitrogen atom and the remaining two carbon atoms are

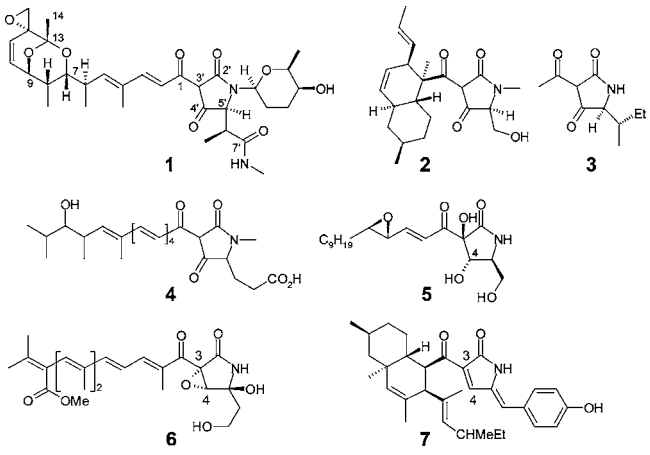


Figure 1. Acyltetramic acids and related compounds: streptolydigin (**1**), equisetin (**2**), tenuazonic acid (**3**), lipomycin (**4**), pramanicin (**5**), fusarin C (**6**), and talaroconvolutin (**7**).

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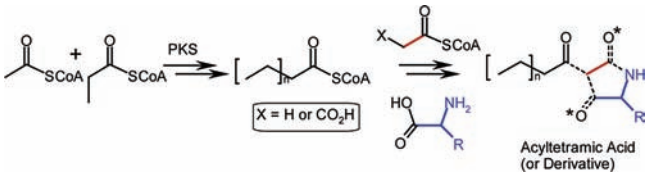
Scheme 1. Overview of Acyltetramic Acid Assembly[a]

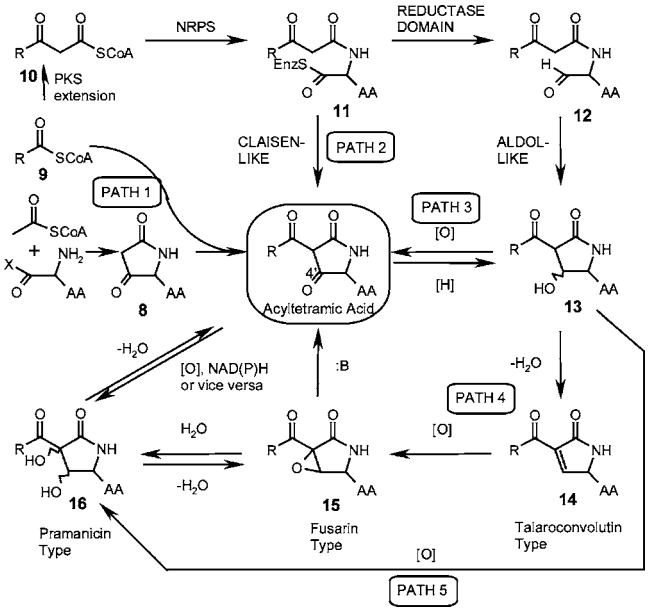


[a] The origin of oxygen atoms O* is addressed in the present work.

derived from an amino acid (Scheme 1).[12] However, the order and indeed the nature of the specific events in which these three moieties condense have remained speculative.

Our previous biosynthetic incorporation experiments into **1**[13] and **5**[14] established related biosynthetic pathways in which a polyketide and an amino acid cyclize to form the pyrrolidinedione ring. The ring C_2 unit in **5** appeared to be derived from acetate, rather than from malonate, a result that suggested a biosynthetic sequence involving acylation of a tetramic acid, i.e., via acylation of **8a** with side chain derivative **9a** (path 1 in Scheme 2). However, the ring C_2

Scheme 2. Putative Pathways Leading to Acyltetramic Acids (e.g., **1**−**4**) and Reactions Connecting ATAs to and from Related Structures (e.g., **5**−**7**)[a]



[a] **a**: Pramanicin. **b**: Streptolydigin. **c**: Equisetin. **d**: Lipomycin. **e**: Fusarin C. For R and the AA side chain, see Figure 1.

unit in **1** (C-2′ and C-3′) was derived from malonate. This result supported path 2 (Scheme 2), where the side chain acyl moiety (**9b**) is extended with a malonate unit on the polyketide synthase (PKS), giving **10b**. Evidence for the intermediacy of **10b** has recently also been obtained using LC/MS by Yuan and co-workers.[15] Attachment of the amino acid (suggested to be β-methylaspartate in **1**[10,11]) ensues, perhaps via **11b**, and cyclization of the resulting carboxylic acid or derivative in a Claisen-like process was suggested to furnish the ATA ring, leading ultimately to **1**.

Recently, Schmidt and co-workers examined the biosynthetic gene cluster for the ATA equisetin (**2**).[16] It was shown that an iterative PKS assembles the acyl side chain (**9c** in Scheme 2), and it was reasoned that this component also then attaches the two-carbon group of the ring, giving **10c**. Adjacent to the PKS is a nonribosomal peptide synthetase (NRPS), which was assigned the function of attaching the requisite amino acid (serine in **2**) to the polyketide via N-acylation; i.e., **10c** gives **11c**. Interestingly, the sequence contains a domain that is homologous with reductive domains in other clusters, suggesting that **11c** would then afford aldehyde **12c**, which in turn would undergo an aldol-like cyclization to afford a 3-acyl-4-hydroxy-pyrrolidinone (**13c**) as the immediate product. Finally, oxidation of **13c** would yield **2** (Scheme 2, path 3). By contrast, a reductase domain was not found in the gene cluster for biosynthesis of lipomycin (**4**), implying pathway 2 (Scheme 2), i.e. direct Claisen-like cyclization of **11d** to **4**.[17]

Cox and co-workers found that the cluster for fusarin C (**6**) also contained a hybrid PKS−NRPS assembly system with a putative reductase at the 3′ terminus.[18] They suggested that the aldehyde intermediate **12e** would cyclize as for **2**, but then elimination of water from the resulting intermediate **13e** would give rise to the α,β-unsaturated system **14e**, which corresponds to the ring functionality in talaroconvolutin (**7**). Epoxidation would then generate the fusarin ring system (**15e**). For the formation of ATAs, it was also suggested that opening of an epoxide analogous to fusarin (i.e., **15**) could create diol **16**, corresponding to the structure found in pramanicin (**5**). The diol could then eliminate water to give the ATA system (Scheme 2, path 4). Other biochemically reasonable reactions that might interconnect these metabolites are also shown in Scheme 2: oxidation of **13** to **16**; base-promoted rearrangement of **15** to give the ATA; conversion of **16** to **15** through dehydration; and formation of **16** from the ATA via oxidation of the enol(ate) and reduction of the ring ketone (C-4′ in **1**).

There are clearly a number of mechanisms that may give rise to the ATAs and related metabolites. The attractive

(9) For recent examples, see: (a) Aflastatin: Ono, M.; Sakuda, S.; Ikeda, H.; Furihata, K.; Nakayama, J.; Suzuki, A.; Isogai, A. *J. Antibiot.* **1998**, *51*, 1019. (b) Trichosetin: Marfori, E. C.; Bamba, T.; Kajiyama, S.; Fukusaki, E.; Kobayashi, A. *Tetrahedron* **2002**, *58*, 6655.

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(11) Pearce, C. J.; Rinehart, K. L., Jr. *J. Antibiot.* **1983**, *36*, 1536.

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mechanism via putative intermediates **14** and/or **15** implies that the ketonic oxygen at C-4′ of the pyrrolidinedione ring in **1** would derive from O_2 or water, whereas the more direct mechanisms (paths 1, 2, or 3) predict that the C-4′ oxygen would be retained from the amino acid. In the case of pramanicin, we used the ^{18}O-induced isotope shift method to show that the oxygen at C-4 was derived intact from the carboxy oxygen of serine, a result consistent with passage through a species such as **11** or **13** but *not* through **14** or **15**.[14]

Therefore, we decided to examine the origin of the oxygen atoms of **1** using the ^{18}O-induced isotope shift method.[19] The results of these experiments and the implications for the alternative mechanisms of ATA cyclization are discussed herein. These results also reveal other features of the biosynthetic pathway, including the mechanism of formation of the bicyclic acetal.

Sodium [1-^{13}C,$^{18}O_2$]acetate and sodium [1-^{13}C,$^{18}O_2$]-propionate were prepared according to literature procedures.[20] [1-^{13}C,1,1,5,5-$^{18}O_4$]Glutamic acid was prepared by acid-catalyzed exchange of DL-[1-^{13}C]glutamic acid with [^{18}O]-water.[21] ESI-MS of the product showed 70% of ^{18}O at each of the four sites.[22] Samples of each precursor were separately administered to growing cultures of *Streptomyces lydicus* ATCC 25470,[23] and streptolydigin was isolated and purified as previously described.[13]

Incorporation of [1-^{13}C,$^{18}O_2$]acetate gave isotopically shifted signals in the ^{13}C NMR spectrum of the resulting **1**, consistent with the presence of ^{18}O at positions C-1, C-9, and C-2′ (Figure 2A, Table 1) but not at C-13. The

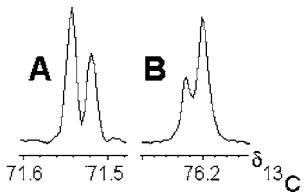

Figure 2. Carbon-13 NMR resonances for the following. (A) C-9 of **1** derived from sodium [1-^{13}C,$^{18}O_2$]acetate. The ^{18}O-shifted signal is on the right; the isotope shift ($\Delta\delta$) is 22 ppb. (B) C-7 of **1** from sodium [1-^{13}C,$^{18}O_2$]propionate; $\Delta\delta$ is 26 ppb.

incorporation levels at these three sites were almost identical, indicative of the same origin for each C_2 unit. The labeling experiment with [1-^{13}C,$^{18}O_2$]propionate showed incorporation of ^{18}O at C-7 only; thus, the bond between C-7 and the oxygen atom bridging C-7 to C-13 in **1** remains intact from propionate (Figure 2B, Table 1).

Table 1. Isotope Shifts and Oxygen-18 Enrichments Observed in Incorporation Experiments with ^{18}O-Labeled Precursors into **1**

carbon no.[a]	precursor[b]	$\Delta\delta$ (ppb)[c]	$^{18}O/^{16}O$[d]
1	acetate	18	0.7
9	acetate	22	0.7
2′	acetate	26	0.7
7′	acetate	33	0.2
7	propionate	26	1.9
4′	glutamate	33	0.2

[a] For the numbering system, see Figure 1. [b] Precursor used in experiment. [c] Isotope shift measured at the specified site when the specified precursor was incorporated. [d] Ratio of intensities of the downfield to the upfield shifted signals.

During the experiment with [1-^{13}C,$^{18}O_2$]acetate, an isotopically shifted resonance was also observed at C-7′. This result is consistent with incorporation through the tricarboxylic acid cycle (TCA), as discussed in detail in our prior manuscript.[13] Thus, it was proposed that acetate incorporates into 2-oxoglutarate which exits the TCA to give glutamate. Glutamate mutase then generates β-methylaspartate, which acts as the direct amino acid precursor for C-4′−C-7′ in **1**. In accordance with this hypothesis, C-7′ exhibits an ^{18}O-induced isotope shift in the sample of **1** from [1-^{13}C,$^{18}O_2$]-acetate, but with some dilution of ^{18}O relative to C-1, C-9, and C-2′ due to passage through one turn of the TCA (Table 1). [1-^{13}C]Acetate also labeled C-4′, although this labeling requires two turns of the TCA. In the current experiments with [1-^{13}C,$^{18}O_2$]acetate, no ^{18}O was observed at C-4′, despite significant carbon-13 enrichment at this site, presumably as the result of extensive exchange and isotopic dilution of oxygen during the longer passage through the TCA. It was therefore not possible to determine the origin of O-4′ in this experiment.

Because the origin of O-4′ was of biosynthetic interest, we therefore explored whether it was derived from glutamate. Initial incorporation experiments with [1,2-$^{13}C_2$]glutamic acid showed doublets surrounding the natural abundance singlets at C-4′ and C-5′, implying that this bond is incorporated as an intact unit and suggesting that glutamate is the direct precursor for the β-methylaspartate moiety of **1**. Carbon-13 enrichment was also detected at other carbon sites in **1** in this experiment, but these signals were enhanced singlets, as opposed to the coupled doublets at C-4′ and C-5′. This result was in accordance with glutamate entering the TCA, which, in combination with the acetate results above, shows that there are efficient and reversible entry and exit from the TCA via interconversion of glutamate and 2-oxoglutarate in this organism. Nonetheless, intact incorporation at C-4′−C-5′ implies that this unit is labeled directly through the action of glutamate mutase, *without* the intervention of the TCA, because the conversion of 2-ketoglutarate to succinate and thence to the polyketide precursors for **1** involves loss of C-1 as carbon dioxide and hence cleavage of the C-1−C-2 bond of glutamate.

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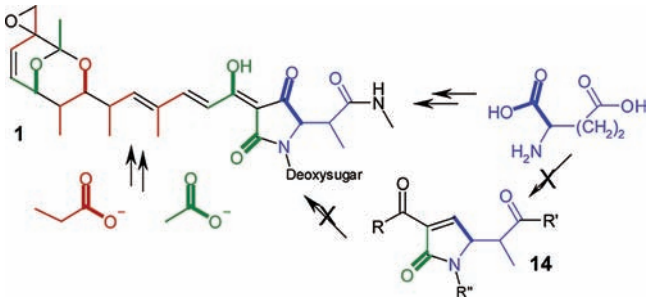
(21) Murphy, R. C.; Clay, K. L. *Biomed. Mass Spectrom.* **1979**, *6*, 309.
(22) The remaining 30%/site was oxygen-16 from the original glutamate and the concentrated aqueous HCl that was used as catalyst.
(23) Sodium acetate and propionate (0.5 g) were administered as solutions in water (3.6 mL); isotopically labeled glutamic acid samples were dissolved in a minimal amount of aqueous HCl. Each solution was added to cultures of *S. lydicus* as a sterile solution every 12 h from 24 h postinoculation until 72 h. Workup was performed after 96 h.

The results of the incorporation experiment with [1-^{13}C,$^{18}O_4$]-glutamate showed incorporation of carbon-13 with an ^{18}O-induced isotopically shifted signal into the 4′ position of the tetramic acid ring (Table 1). As the oxygen is retained from the precursor, the amino acid does not go through the TCA, where C-1 and the attached oxygen atoms would be lost as CO_2. Instead, as for the [$^{13}C_2$]glutamate case above, the glutamate must undergo glutamate mutase-catalyzed rearrangement to [1-^{13}C,$^{18}O_4$]β-methylaspartate directly, which is then incorporated into **1** with retention of oxygen-18. It is thus apparent that the route to the tetramic acid ring in **1** cannot involve loss of O-4′ from the amino acid precursor. Therefore, intermediates such as **14** or **15** cannot be on the direct path to the natural product (Scheme 3). Further, if there

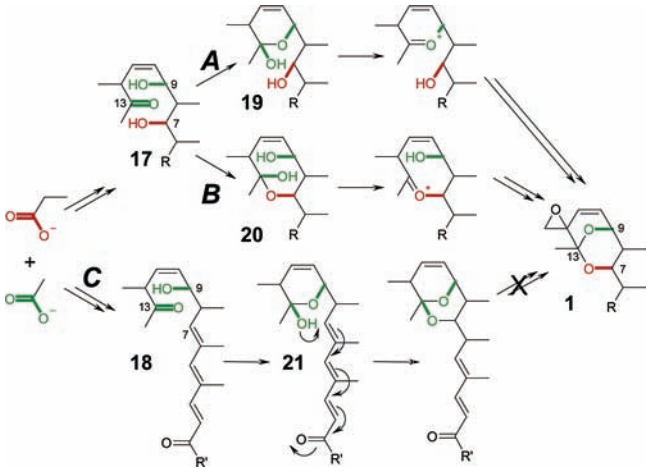
Scheme 3. Summary of Labeling Results



is a common pathway to the ATAs and related natural products, then either **13** or the ATA is a likely common precursor structure, in accord with the proposed paths 2 or 3. Natural products such as **5**−**7** would then be derivatives that branch from this generic precursor structure, as opposed to intermediates in the ATA assembly sequence. Experiments are now underway to elucidate further the details of these processes.

The results also shed light on the mechanism for formation of the bicyclic ketal moiety in **1**. This group could be formed via at least three different pathways from putative polyketide precursors **17** or **18**, bearing a ketone at C-13 (Scheme 4). In pathway A, the C-9 hydroxy group of **17** attacks C-13 to form hemi-ketal **19**; loss of water and attack of the 7-hydroxy group on the ensuing oxonium ion give the ketal. In pathway B, hemi-ketal **20** is formed; cyclization with the 9-OH group gives the ketal. In pathways A and B, the oxygen at C-7 in **1** should originate from propionate. In Pathway C, the 9-OH group of **18** attacks C-13 to form hemi-ketal **21**, the hydroxyl group of which then adds to the alkene at C-7. This latter route is attractive because it formally involves a conjugate

Scheme 4. Putative Pathways for Formation of the Bicyclic Ketal in **1**[a]



[a] Reaction proceeds through paths A or B but not C.

addition to a polyunsaturated ketone. In this pathway, the oxygen at C-7 originates from the C-13 acetate unit and *not* from propionate.

The observation of an isotope shift at C-7 in **1** from [1-^{13}C,$^{18}O_2$]propionate and the lack of a shift at C-13 in **1** derived from acetate exclude path C in the biosynthesis of **1** but are consistent with paths A or B. Isotopic labeling experiments cannot distinguish the latter two possibilities. Perhaps the process prefers pathway A, as the six-membered ring in **19** may be favored over the larger ring in **20**.

In summary, results with ^{18}O-labeled precursors restrict the number of putative pathways to streptolydigin and by implication potentially to other ATAs. They specifically exclude a pathway via enamide **14b** and epoxide **15b**. The data compliment and extend the recent gene cluster analyses of these metabolites. Further, the bicyclic ketal in **1** is formed by a process that resembles the normal mechanism of ketal formation in organic chemistry. It is unknown whether this step is enzyme-catalyzed or spontaneous; this issue will be addressed in the future.

Acknowledgment. We thank the Natural Sciences and Engineering Research Council of Canada for support.

Supporting Information Available: ^{13}C NMR spectra of **1** showing isotope shifts and a table of incorporation data for [1,2-$^{13}C_2$]glutamate. This material is available free of charge via the Internet at http://pubs.acs.org.

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